SEMI-ANNUAL PROGRESS REPORT FOR AWARD NUMBER NA16FX1412 "METAL TOXICITY IN STELLER SEA LION (*EUMETOPIAS JUBATUS*) TISSUES AND CELL LINES"

A. Project Identifiers

Award Number: NA16FX1412
Grant Program/CFDA#: 11.439
Recipient Organization: Yale University
Principal Investigator: John P. Wise, Sr.
Project Title: Metal Toxicity in Steller Sea Lion (*Eumetopias Jubatus*) Tissues and Cell Lines
Funding: Federal: $1,096,715 Match: $_____
Award Period: From: July 1, 2001 To: June 30, 2004
Period Covered by this Report: From: July 1, 2001 To: Dec 31, 2001

B. Project Summary

The western population of the Alaskan Steller sea lion has become endangered and is continuing to decline for unknown reasons. The fact that this decline is limited to this population and not other Steller sea lion populations strongly suggests that there is an environmental factor involved. This proposal investigates the role of contaminants as environmental factors in the decline of the western population of the Steller sea lion. It focuses on metals, a particularly widespread and toxic class of environmental contaminants and measures their accumulation in the tissues of the sea lions. Further, it investigates the toxicity of these metals in the major organ systems of the sea lions by establishing cell lines from these organ systems and determining the effects of metals in these lines, so that a priority list can be developed for intervention measures. Thus this proposal will provide important information regarding environmental contaminants and how they may affect the health of animals involved in the population decline.

In addition to the short-term gain of information that will help in developing a plan to aid the recovery of the Steller sea lion, this proposal has several long-term benefits. First, by creating cell lines of several organs and sharing them with other scientists, this proposal will allow for detailed mechanistic studies of metals and other contaminants to help us further understand and predict potential threats to the Steller sea lion. In addition, these cell lines will allow for more detailed biochemical and physiological studies of Steller sea lion biology, which will aid in maintaining the health of this population. Finally, the cell lines will serve as a model of sea lions and marine mammals in general and allow for comparisons with data in human cells to determine similarities and differences between humans and marine mammals.

Samples will be collected at the Alaska Sea Life Center, The Marine Mammal Center, and Mystic Aquarium. Primary cultures will be derived at those locations and then shipped to Yale for generation of continuous cultures and metal toxicity testing. Continuous cell lines will then be sent to Mystic Aquarium for storage and distribution. Samples of biopsy and autopsy tissues will be sent to Northern Arizona University in frozen form and will be maintained frozen until analysis is commenced. For comparisons between Steller sea lion populations, additional sets of

frozen tissue will be collected from the eastern Alaskan and British Colombian populations. Cell lines will not be derived from these animals

C. Summary of Progress and Results

The short–term goal of this research is to determine the toxicity of metals to Steller sea lions and to prioritize the risk that specific metals represent in order to suggest potential measures for intervention. Specifically, we are investigating the following hypothesis: ***Steller sea lions bioaccumulate metals to toxic levels***. The long-term goal of this research is to use the cell lines established in this work to better understand the cellular biochemistry and physiology of Steller sea lions to ensure their continued recovery.

The first step was to obtain the appropriate permits from the National Marine Fisheries Service to conduct the work. This was accomplished and we were assigned permit #1008-1637-00 with an expiration date of 10/31/06. With the permit in hand we began the scheduled tasks described below.

1. Scheduled Tasks

a. *Specific Aim #1: Determine Tissue Levels of Metals in Steller Sea Lions*

- Begin tissue collection.

b. Specific Aim #2: Establish Cell Lines from Steller Sea Lion Organs

- Obtain skin samples from Steller sea lions at Mystic aquarium and begin primary cultures.
- Begin immortalization of primary cultures

c. Specific Aim #3: Evaluate the Toxicity of Metals in the Cell Lines

- No tasks scheduled for this time period.

d. Specific Aim #4: Distribute the Cell Lines to Other Investigators

- No tasks scheduled for this time period.

2. Activities

a. Specific aim #1: Determine Tissue Levels of Metals in Steller Sea Lions

- Established initial protocols for collection and processing tissue for metal analysis.
- Tissue from one Steller sea lion was received from Alaska and is currently stored at –70°C pending analysis.

b. Specific aim #2: Establish Cell Lines from Steller Sea Lion Organs

- Skin samples were obtained from Steller sea lions in residence at Mystic aquarium.
- Primary cultures were established and characterization of primary cell lines was initiated.
- Immortalization of primary cultures was begun.

c. Specific Aim #3: Evaluate the Toxicity of Metals in the Cell Lines

- No tasks scheduled for this time period

d. Specific Aim #4: Distribute the Cell Lines to Other Investigators

- No tasks scheduled for this time period.

3. Changes to the goals/objectives during this time period

There have been no changes to the goals/objectives of this study.

4. Results and/or specific products prepared during the reporting period.

a. Specific aim #1: Determine Tissue Levels of Metals in Steller Sea Lions

- No results scheduled for this time period.

b. Specific aim #2: Establish Cell Lines from Steller Sea Lion Organs

We began the process of characterizing the skin fibroblast lines (named SSL1, SSL2 and SSL3) for growth parameters and karyotypic features and froze primary cultures for additional future use. We found that Steller sea lions had a normal diploid chromosome number of 36 (Table 1). This is important to establish because some of the metal toxicity testing could involve chromosomal effects and an abnormal karyotype can be indicative of specific disease states. We found that the cells exhibited normal log phase growth (Figures 1 and 2). Importantly, we discovered that Steller sea lion skin fibroblasts senesce after 21 population doublings, which is a short life span in cell culture, but which is consistent with culture life span of cells from other species. It is our expectations that hTERT will significantly alter this life span.

Table 1. Median chromosomes number for Steller sea

Cell line	Diploid Chromosome Number [a]
SSL1	36
SSL2	36
SSL3	36

[a] 50 Metaphase spreads were analyzed for each cell line.

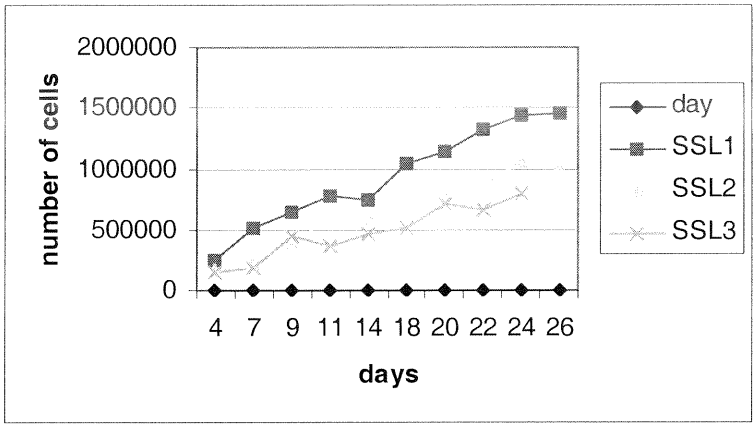

Figure 1. Three growth curves of Steller sea lion skin fibroblasts. Initial cultures were seeded with 50,000 cells at population doubling 10. (Note: SSL3 was significantly older and later euthanized due to a very large tumor)

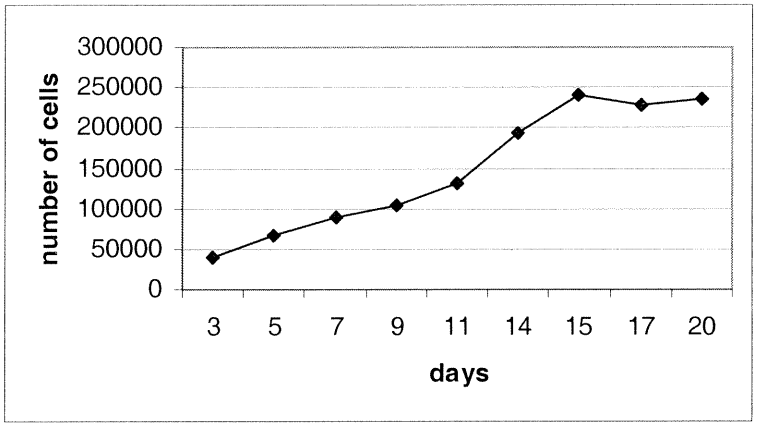

Figure 2. Additional growth curve of SSL1. Initial cultures were seeded with 50,000 cells at population doubling 15. (Sea lion cells were found to senesce at approximately population doubling 21).

Because it was our most rigorous growing cell line, we decided to focus our initial hTERT infections on SSL1. We were able to successfully infect the cells with hTERT, but the expression was very weak suggesting the need for optimizing protocols.

c. *Specific Aim #3: Evaluate the Toxicity of Metals in the Cell Lines*

- No results were scheduled for this time period. Because of delays in Specific Aim #2 we elected to begin going forward with this specific aim early. We first established the plating efficiency of SSL1 for use in the cytotoxicity assay. Table 1 shows the plating efficiency for several cell densities all of which fall in the normal range for mammalian cell cultures. We selected a cell density of 500. Figure 3 shows concentration-dependent cytotoxicity of our first chemical, hexavalent chromium. Concentrations of 1, 2.5, 5 and 10 uM induced 69, 87, 46 and 49 percent relative survival respectively. These assays are currently being repeated.

Table 2. Plating efficiency of SSL1

Cell Density	Average number colonies/dish	Plating efficiency
500	118	23.6%
1000	129.7	13%
2000	190.7	10%
5000	287.7	6%

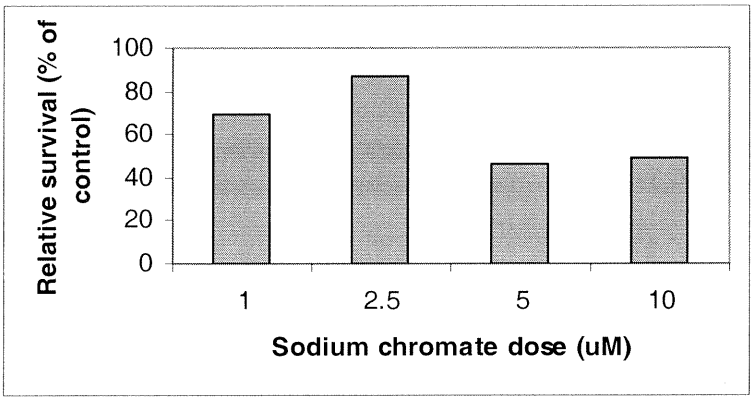

Figure 3. Cytotoxicity of Na_2CrO_4 in Steller sea lion skin fibroblasts. There is concentration-dependant cytotoxicity with some variability due to cellular age.

d. Specific Aim #4: Distribute the Cell Lines to Other Investigators

- No results scheduled for this time period.

D. Problems

1. Circumstances of problems that prevented completion of a scheduled task. Describe consequences resulting from inability to complete a given task.

a. Specific aim #1: Determine Tissue Levels of Metals in Steller Sea Lions

- Limited sources of tissues due to delayed funding, seasonal nature of collection trips, and potential tissue collectors having permit problems.

b. Specific aim #2: Establish Cell Lines from Steller Sea Lion Organs

- We received necropsied tissue from a Steller sea lion at Mystic aquarium. While this animal was a very sick animal it provided us with valuable experience at culturing other types of tissue from other organs, i.e. blubber, tumor, liver, kidney, spleen, ovary, lung, brain, and skin. Because of the non-sterile conditions of collection these cultures all became contaminated.

- We received tissue (skin, bronchi, liver, kidney) from a steller sea lion pup. Attempts were made at dissecting and culturing this tissue, however, this tissue was sent around the time of the

September 11th disaster, as a result it spent four days in transit and was received in very poor condition. There was no success in culturing this tissue.

- Infection of marine mammals yielded weak expression.

- Few samples available.

c. *Specific Aim #3: Evaluate the Toxicity of Metals in the Cell Lines*

- No problems.

d. Specific Aim #4: Distribute the Cell Lines to Other Investigators

- No problems.

2. Actions/activities taken to resolve the problem

a. Specific aim #1: Determine Tissue Levels of Metals in Steller Sea Lions

- Spring season will provide further collection of tissue.
- National Marine Fisheries Services permits will be ready to most potential collectors

b. Specific aim #2: Establish Cell Lines from Steller Sea Lion Organs

- Spring season will provide further collection of tissue.
- Two failures resulted from unique problems that should not occur again.
- Optimizing hTERT infection.
- Creating protocols and training using other marine mammals. Results shown in Table 3.

3. Special problems or differences between budgeted and actual expenditures.

None at this time.

Table 3. Other Marine Mammals used for cell culture.

Species	Tissue type	Source/description	Primary cultures established
Beluga	Dermis	Mystic, resident animal	Yes
Common Dolphin	Dermis	Rehabilitation attempt	Yes
	Kidney		Yes
	Liver		No
	Bronchus		Yes
Bottlenose Dolphin	Muscle	Mystic, sudden unexpected death of resident animal	No
	Bronchus		No
	Brain		No
	Liver		No
	Kidney		No
	Heart		No
	Dermis		Yes
Mink (mink are used as a reproductive model for Steller sea lion)	Dermis	Michigan State University	Yes